PATENT

UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 00-1278)

In the Application of: **Lawton, et al.**

Serial No.: **09/765,739**

Filed: **January 18, 2001**

Art Unit: 1645

Examiner: V. Ford

For: **Compositions and Methods for Detection of *Ehrlichia canis* and *Ehrlichia chaffeensis* Antibodies**

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Ramaswamy Chandrashekar, am a research scientist for IDEXX Laboratories, Inc., the entire Assignee of U.S. Pat. Appl. Ser. Nos. 09/765,739, 10/054,354, and 10/054,647. I have earned a Ph.D. in Biochemistry and M.S. in Zoology. I have performed research and development in the field of sero-diagnostics for veterinary bacterial pathogens, including, for example, *Ehrlichia* ssp. and *Streptococcus equi* for over five years. In addition, I have performed research and development in the field of sero-diagnostics for nematode infections in both humans and animals for over 20 years. I am an author of over 70 scientific publications in the field of diagnosis and prevention of nematode infections. A copy of my Curriculum Vitae is attached.

2. Waner *et al., (J. Vet. Diagn. Invest.*, Vol. 12, pp. 240-244, 2000), Cadman *et al., (The Veterinary Record,* 135, 362), Rikihisa (WO 99/13720), and other references

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that teach or suggest the use of entire *E. canis* or *E. chaffeensis* infected cells, whole (*i.e.,* non-fragmented) *E. canis* or *E. chaffeensis* proteins, including mixtures of whole proteins, natural whole proteins, or whole recombinant proteins, to, *e.g.*, detect *Ehrlichia,* do not teach or suggest polypeptides of SEQ ID NOs:1-7 to, *e.g.*, detect *Ehrlichia.* As explained in the specifications of the above-mentioned patent applications, entire *E. canis or E. chaffeensis* infected cells, or whole (i.e., non-fragmented) *E. canis* or *E. chaffeensis* proteins, including mixtures of whole proteins, natural whole proteins or whole recombinant proteins are impure reagents, which are of limited usefulness in sero-diagnosis due to sensitivity and specificity issues. For instance, Example 1 of the 09/765,739 application demonstrates that assays that use SEQ ID NOs:1 and 2 were more sensitive and specific than assays that use partially purified *E. canis* antigens. *See e.g.*, paragraph spanning page 20 and 21 of the 09/765,739 application. The partially purified *E. canis* antigens were obtained from *E. canis* organisms grown in tissue culture and partially purified by differential centrifugation and column chromatography. These partially purified *E. canis* antigens were therefore, mixtures of whole proteins.

3. Assays for detecting anti-*Ehrlichia* antibodies or fragments as described by Waner, Cadman, Rikihisa, and others are severely limited in usefulness because of sensitivity and specificity issues directly related to the impure nature of the *Ehrlichia* antigen used in these tests. *See e.g.*, page 2, line 21 through page 3, line 2 of the 09/765,739 specification (emphasis added). The instant inventions provide highly purified reagents for the detection *Ehrlichia*, that is, polypeptides of about 18-20 amino acids. The use of SEQ ID NOs:1-7 instead of the impure reagents described above, to for

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example, detect *Ehrlichia* provide distinct advantages such as greater sensitivity and specificity in sero-diagnostic assays.

4. Waner teaches that the disclosed ELISA assay for detection of *E. canis* closely correlates to the "gold standard" IFA test. *See e.g.*, p. 243, left col. first full paragraph; page 243, right col., first and second paragraph; page 240, right col., first full paragraph.

5. Cadman teaches that the disclosed dot-blot enzyme linked immunoassay (DBELIA) had a sensitivity of 92% and a specificity of 96% when compared to the IFA. See page 135, paragraph spanning columns. Cadman states that the "study showed the DBELIA to be as sensitive and specific as IFA for the detection of antibodies to *E. canis*." See last paragraph.

6. The polypeptides claimed in the instant application have a sensitivity of 98.5% and a specificity of 100% when compared to western blot analysis. Western blot analysis is more sensitive and more specific than IFA analysis. The IFA had, at one time, been considered the "gold standard" for sero-diagnosis of *Ehrlichia*. However, western analysis is more sensitive and more specific than IFA analysis, which uses whole cells as the antigen resulting in cross-reactivity, specificity, and sensitivity issues. The IFA disclosed in the instant invention had a sensitivity of 88% and a specificity of 0%. The polypeptides of the instant invention perform better than the "gold standard" IFA in this study. The Waner and Cadman assays, however, perform only as well as the IFA. Therefore, one of skill in the art could reasonably conclude that the polypeptides of the instant invention perform better, *i.e.*, provide more sensitive and specific results in sero-diagnostic assays, than the Waner and Cadman assays.

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7. Rikihisa teaches the use of recombinant, whole proteins to detect *Ehrlichia* antibodies. Rikihisa does not disclose the sensitivity or specificity of the whole, recombinant proteins in sero-diagnostic assays. However, Ohashi *et al.* (J. Clin. Microbiol. 36:2671 (1998)) (copy attached) teaches that dot blot assays performed with whole *E. canis* rP30 antigen to detect *E. canis* were as sensitive as an IFA assay, specificity was not examined in this study. See page 2678, right column, first full paragraph. The instant invention provides peptides (SEQ ID NOs:1-7) that can provide results that are more sensitive than IFA assays. Therefore, one of skill in the art could reasonably conclude that the peptides of the instant invention are more sensitive and more specific than the antigens reported in Waner, Cadman, and are more sensitive than the antigens reported in Rikihisa.

8. The pure reagents described in the instant inventions have additional advantages as compared to the impure reagents described in Waner, Cadman, and Rikihisa. For example, in experiments performed at IDEXX Laboratories mixtures of SEQ ID NOs:1 and 2 showed no cross-reactivity to *Borrelia burgdorferi, A. phagocytophilum*, and uninfected canine serum. See Table 1.

Table 1.

Peptide	**# of Samples**	**Canine Serum**	**Reactivity**
Mixtures of SEQ ID NO:1 and SEQ ID NO:2	157	Uninfected	0/157
	81	*E. canis*	81/81
	166	*Borrelia burgdorferi*	0/166
	29	*A. phagocytophilum*	0/29

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9. I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 02/13/2004 By: _____

Ramaswamy Chandrashekar

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PROFESSIONAL EXPERIENCE

IDEXX LABORATORIES, WESTBROOK, MAINE

RESEARCH SCIENTIST-II, RAPID ASSAY GROUP, INFECTIOUS DISEASES R&D (2002-PRESENT)

Leading a group responsible for R&D of Point Of Care of Testing devices for companion animals.

HESKA CORPORATION, FORT COLLINS, COLORADO

SENIOR SCIENTIST AND GROUP LEADER, Diagnostic Research (1998-2002)

Management of molecular- and immuno-diagnostic group. Supervised a group of five Scientists (including two Ph.D. Scientists). Designed, developed, and evaluated molecular and immunodiagnostic assays for the diagnosis of parasitic and infectious agents in companion animals. Developed, optimized, and validated immunoassays for cancer markers. Project management: primary person responsible for the management and coordination of projects under aggressive time-lines. Responsible for transfer of assays, documentation and SOPs. Interacted extensively with groups with in the company (process and regulatory groups) and outside the organization (external scientific/technical collaborations with both academia and industry).

ACCOMPLISHMENTS

Participated in the research and/or development of the following *Heska* products and reagents:

- ***Heska™* Canine *Ehrlichia* sp. PCR Test** (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- ***Heska™* Feline ImmuCheck™ Assay** (Vaccine Titer Assay) (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- ***Heska™ Equine Streptococcus equi* PCR Test** (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- ***Heska™* Canine Heartworm Antigen Test** (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- ***Heska™* SoloStep™ CH** (USDA Licensed Canine Heartworm Diagnostic POCT).
- ***Heska™* SoloStep™ FH** (USDA Licensed Feline Heartworm Diagnostic POCT).
- Seven patents pending.
- Identified several potential diagnostic targets for a fecal diagnostic kit for intestinal helminth infections.

SENIOR SCIENTIST, Nematode Vaccines and Diagnostics (1995-1998)

Management of vaccine antigen discovery and testing group. Supervised one post-doctoral and five associate scientists. Conducted basic research on the biology/ biochemistry/immunology/proteomics of parasitic nematodes to identify potential vaccine antigens. Molecular cloning and expression of recombinant nematode vaccine antigens. Designed and coordinated several animal studies for vaccine efficacy trials (dogs and cats). Antigen discovery research for heartworm diagnostics.

ACCOMPLISHMENTS

- Identified and characterized of over ten potential vaccine candidate nematode antigens against heartworm infections for clinical trials in dogs/cats.
- Five potential recombinant antigens identified by proteomics and cloned for diagnostic evaluation in antibody detection ELISA for feline heartworm infections.
- 13 Patents issued and one pending.

WASHINGTON UNIVERSITY, ST. LOUIS, MISSOURI

RESEARCH ASSISTANT PROFESSOR, Dept. of Medicine and Molecular Microbiology (1995)
RESEARCH INSTRUCTOR, Dept. of Medicine and Molecular Microbiology (1991-1995)

Responsible for immunodiagnostic assay development and vaccine discovery studies in parasitic nematode infections. Field evaluation of diagnostic tests in Egypt. Supervised science technicians.

ACCOMPLISHMENTS

- Developed recombinant antigen-based antibody ELISAs for the diagnosis of human nematode infections. Evaluation of both assays extensively in field studies in Africa.
- Developed a monoclonal antibody-based antigen-based diagnostic assay for human onchocerciasis (river blindness) to detect circulating parasite antigens both in blood and urine.
- Developed a recombinant antigen-based immunoblot assay for diagnosis of Histoplasmosis.
- Identified and tested several candidate recombinant vaccine antigens in animal model of filarial nematode infections.
- One Patent issued and one pending.

NATIONAL/INTERNATIONAL SCIENTIFIC ACTIVITIES

- *Invited Participant-WHO:* Filariasis Scientific Working Group (UNDP/World Bank/World Health Organization), 1991, 1994. "Protective immunity studies in Onchocerciasis.
- *Chairperson:* Chaired the scientific session in Filarial Biology. Joint Annual Meeting of the American Society of Tropical Medicine and Hygiene and The American Society of Parasitologists, Atlanta, Georgia. October 31-November 4, 1993.
- *Advisor-WHO:* Special Program for Research & Training in Tropical Diseases (TDR), World Health Organization, 1993, 1994. Transferred technology from lab research to product development.
- *Consultant and collaborating scientist*, Epidemiology and Control of Vector Borne Diseases in the Middle East (Egypt-Israel-USA) (USAID/NIH), 1990-1994. Transferred immunodiagnostic assays for lymphatic filariasis from laboratory to field for evaluation studies in Egypt.
- *Collaborating Scientist,* Participated in protective immunity studies in human filariasis-*International Collaborations in Infectious Disease Research Project (NIH)* to study immunity to filariasis in humans with a longitudinal study of carefully defined populations in a highly endemic region of Egypt, 1994-1995.

POST-DOCTORAL RESEARCH ASSOCIATE (Jewish Hospital of St. Louis at Washington University) (1988-1991)

Identified, characterized and generated monoclonal antibodies to circulating parasitic nematode antigens; Developed antigen detection and recombinant antigen-based antibody assays for human infectious diseases. Participated in a Recombinant DNA Technology Workshop conducted by the *New England Biolabs* and Smith College, Northampton (1991). Supervised a science technician.

CIBA-GEIGY RESEARCH CENTER, BOMBAY, INDIA

SENIOR RESEARCH ASSISTANT (1986-1988)

RESEARCH ASSISTANT (1980-1986)

EDUCATION

Ph.D., Biochemistry - CIBA-GEIGY Research Center, and University of Bombay, India.

MS., Zoology - University of Madras, Madras, India.

HONORS AND AWARDS

National Science Talent Search Scholarship, NCERT, New Delhi, India.
Joshua Gold Medal for best under graduate student.
Joshua Gold Medal and Aaron award for best post graduate student.
National Merit Scholarship, Government of India.

PROFESSIONAL MEMBERSHIPS

American Society of Tropical Medicine and Hygiene.
American Association for the Advancement of Science.
American Society of Parasitologists.
American Society for Microbiologists.

PUBLICATIONS

(SEE ADDENDUM)

Peer-reviewed-64; Invited-7

ADDENDUM

PUBLICATIONS

PEER-REVIEWED

1. Rao RR, Marathe MR, **Chandrashekar R**, Subrahmanyam D: Ocular filarial infections in *Mastomys natalensis* with *Litomosoides carinii* and *Brugia pahangi*. *Indian J Parasitol* 1983;7:57-60.
2. Reddy AB, Rao UR, **Chandrashekar R**, Shrivastava R, Subrahmanyam D: Comparative efficacy of some benzimidazoles and amoscanate (Go. 9333) against experimental filarial infections. *Tropenmed Parasitol* (Germany) 1983; **34**:259-262.
3. **Chandrashekar R**, Rao UR, Rajasekariah GR, Subrahmanyam D: Separation of viable microfilariae free of blood cells on Percoll gradients. *J Helminthol* 1984;**58**:69-70.
4. **Chandrashekar R**, Rao UR, Subrahmanyam D, Hopper K, Nelson DS, King M: *Brugia pahangi*: Serum-dependent cell-mediated reactions to sheathed and exsheathed microfilariae. *Immunology* 1984;**53**:411-417.
5. **Chandrashekar R**, Rao UR, Rajasekariah GR, Subrahmanyam D: Isolation of microfilariae from blood on iso-osmotic Percoll gradients. *Indian J Med Res* 1984;**79**:497-501.
6. **Chandrashekar R**, Rao UR, Subrahmanyam D: Effect of diethylcarbamazine on serum dependent cell-mediated reactions to microfilariae in vitro. *Tropenmed Parasitol* (Germany) 1984;**35**:177-182.
7. **Chandrashekar R**, Parab PB, Subrahmanyam D: The effect of p-amino-benzoic acid and folic acid on the development of infective larvae of *Brugia malayi* in *Aedes aegypti*. *Acta Trop* (Switzerland) 1984;**41**:61-67.
8. Reddy AB, **Chandrashekar R**, Rao UR, Subrahmanyam D: Microfilarial periodicity in *Mastomys natalensis*. *J Helminthol* 1984;**58**:117-121.
9. Hopper K, Subrahmanyam D, Gregory J, Nelson DS, Rao UR, **Chandrashekar R**: Mechanisms of ADCC of rat cells to microfilariae: effects of metabolic inhibitors and electron microscopic observations. *Asian Pac J Allergy Immunol* 1984;**2**:17-21.
10. **Chandrashekar R**, Rao UR, Subrahmanyam D, Hopper K, Nelson DS, King M: Immune reactions to exsheathed microfilariae of *Litomosoides carinii*. *Indian J Med Res* 1985;**81**:260-268.
11. **Chandrashekar R**, Rao UR, Subrahmanyam D: Sharing of antigens among filarial species in antibody-dependent cell-mediated cytotoxicity. *J Biosci* (India) 1985;**9**:191-196.
12. **Chandrashekar R**, Rao UR, Parab PB, Subrahmanyam D: *Brugia malayi*: Cell-mediated immune reactions to microfilariae. *Southeast Asian J Trop Med Public Health* 1985;**16**:15-21.
13. **Chandrashekar R**, Rao UR, Subrahmanyam D: Serum-dependent cell-mediated immune reactions to *Brugia pahangi* infective larvae. *Parasite Immunol* 1985;**7**:633-641.
14. **Chandrashekar R**, Rao UR, Arab PB, Subrahmanyam D: *Brugia malayi*: Rat-cellular interactions with infective larvae mediated by complement. *Exp Parasitol* 1986;**62**:362-369.
15. Rao UR, **Chandrashekar R**, Parab PB, Rajasekariah GR, Subrahmanyam D: Lectin-binding characteristics of *Wuchereria bancrofti* microfilariae. *Acta Trop* (Switzerland) 1986;**44**:35-42.
16. Rao UR, **Chandrashekar R**, Subrahmanyam D: *Litomosoides carinii*: Characterization of surface carbohydrates of microfilariae and infective larvae. *Tropenmed Parasitol* (Germany) 1986;**38**:15-18.
17. Rao UR, **Chandrashekar R**, Subrahmanyam D: Effect of Ivermectin on serum-dependent cellular interactions to *Dipetalonema viteae* microfilariae. *Tropenmed Parasitol* (Germany) 1986;**38**:123-126.
18. Rao UR, **Chandrashekar R**, Rajasekariah GR, Subrahmanyam D: Wheatgerm agglutinin binds to infective larvae of *Wuchereria bancrofti* but not to those of *Brugia malayi* and *Brugia pahangi*. *J Parasitol* 1987;**73**:1256-1258.
19. Rao UR, **Chandrashekar R**, Subrahmanyam D: Complement activation by eggs and microfilariae of filarial parasites. *Immunol Cell Biol* (Australia) 1987;**65**:365-370.
20. Rao UR, **Chandrashekar R**, Subrahmanyam D: Developmental changes in surface carbohydrates of filariae. *Indian J Med Res* 1988;**87**:9-14.
21. Parab PB, Rajasekariah GR, **Chandrashekar R**, Alkan SS, Subrahmanyam D: Characterization of a monoclonal antibody specific to the infective larvae of *Brugia malayi*. *Immunology* 1988;**64**:169-174.
22. Rajasekariah GR, Mukherjee P, **Chandrashekar R**, Subrahmanyam D: *Brugia pahangi*: Clearance of circulating microfilariae in immunized mice. *Immunol Cell Biol* (Australia) 1988;**66**:331-336.
23. Rajasekariah GR, Deshpande L, Parab PB, **Chandrashekar R**, Subrahmanyam D: *Wuchereria bancrofti* larvae in naturally infected *Culex quinquefaciatus*. *Ann Trop Med Parasitol* (Liverpool) 1988;**82**:637-639.
24. **Chandrashekar R**, Yates JA, Weil GJ: Use of parasite antigen detection to monitor macrofilaricidal therapy in *Brugia malayi* infected jirds. *J Parasitol* 1990;**76**:122-124.
25. **Chandrashekar R**, Rao UR, Subrahmanyam D: IgG response of rats to excretory-secretory products of *Litomosoides carinii*. *Parasitol Res* (Germany) 1990;**76**:420-423.
26. **Chandrashekar R**, Rao UR, Subrahmanyam D: Immune response to *Acanthocheilonema viteae* infection in multimammate rat (*Mastomys natalensis*). *Immunol Cell Biol* (Australia) 1990;**68**:21-26.
27. Weil GJ, **Chandrashekar R**, Liftis F, McVay CS, Bosshardt SC, Klei TR: Circulating parasite antigen in *Brugia pahangi* infected jirds. *J Parasitol* 1990;**76**:78-84.
28. Weil GJ, Ogunrinade AF, **Chandrashekar R**, Kale OO: IgG_4 subclass serology for onchocerciasis. *J Infect Dis* 1990;**161**:549-554.
29. **Chandrashekar R**, Rao UR, Subrahmanyam D: Antibody-mediated cytotoxic effects *in vitro* and *in vivo* of rat cells on infective larvae of *Brugia malayi*. *Int J Parasitol* 1990; **20**:725-730.

30. **Chandrashekar R**, Ogunrinade AF, Alvarez RM, Kale OO, Weil GJ: Circulating immune-complex associated parasite antigens in human onchocerciasis. *J Infect Dis* 1990;**162**:1159-1162.
31. Rao UR, **Chandrashekar R**, Subrahmanyam D: Effect of Ivermectin on filarial infections of *Mastomys natalensis*. *Parasitol Res* (Germany) 1990;**76**:521-526.
32. **Chandrashekar R**, Subrahmanyam D, Weil GJ: Effect of CGP 20376 on *Brugia malayi* and parasite antigenemia in jirds. *J Parasitol* 1991;**77**:479-482.
33. **Chandrashekar R**, Masood K, Alvarez RM, Ogunrinade AF, Lujan R, Richards FO, Weil GJ: Molecular cloning and characterization of recombinant parasite antigens for immunodiagnosis of onchocerciasis. *J Clin Invest* 1991;**88**:1460-1466.
34. Li BW, **Chandrashekar R**, Alvarez RM, Liftis F, Weil GJ: Identification of paramyosin as a potential protective antigen against *Brugia malayi* infection in jirds. *Mol Biochem Parasitol* 1991;**49**:315-324.
35. Rao UR, **Chandrashekar R**, Subrahmanyam D: Cell-mediated cytotoxicity of sera from diethylcarbamazine- and Ivermectin-treated rats on microfilariae *in vitro*. *Trop Biomed* 1991;**8**:1-6.
36. Rajasekariah GR, Parab PB, **Chandrashekar R**, Deshpande L, Subrahmanyam D: Pattern of *Wuchereria bancrofti* microfilaremia in young adolescent school students in Bassein, an endemic area for lymphatic filariasis. *Ann Trop Med Parasitol* (Liverpool)1991;**85**:663-666.
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38. Weil GJ, Li BW, Liftis F, **Chandrashekar R**: *Brugia malayi*: Antibody responses to larval antigens in infected and immunized jirds. *Exp Parasitol* 1992;**74**:315-323.
39. Ogunrinade AF, **Chandrashekar R**, Weil GJ, Kale OO: Use of a recombinant antigen (OC 3.6) for the serological diagnosis of onchocerciasis in exposed Nigerian children. *J Trop Pediatr* (London) 1992;**38**:103-105.
40. Li BW, **Chandrashekar R**, Weil GJ: Vaccination with recombinant filarial paramyosin induces partial immunity to *Brugia malayi* infection in jirds. *J Immunol* 1993;**150**:1881-1885.
41. Ogunrinade AF, **Chandrashekar R**, Eberhard ML, Weil GJ: Preliminary evaluation of recombinant *Onchocerca volvulus* antigens for serodiagnosis of onchocerciasis. *J Clin Microbiol* 1993; **31**:1741-45.
42. **Chandrashekar R**, Ogunrinade AF, Henry RW, Lustigman S, Weil GJ: *Onchocerca volvulus*: Characterization of monoclonal antibodies to immune complex-associated parasite antigens. *Exp Parasitol* 1993; **77**:224-234.
43. **Chandrashekar R**, Curtis KC, Ramzy RM, Liftis F, Li B-W, Weil GJ: Molecular cloning of *Brugia malayi* antigens for diagnosis of lymphatic filariasis. *Mol Biochem Parasitol* 1993; **64**:261-271.
44. **Chandrashekar R**, Curtis KC, Weil GJ. Molecular characterization of a circulating parasite antigen in sera fromonchocerciasis patients that is immunologically cross-reactive with human keratin. *J Infect Dis* 1995; **171**:1586-1592.
45. Eberhard ML, Tsang VC, Ottesen EA, **Chandrashekar R**, Weil GJ, Dickerson JW, Walker EM. Comparison of parasitologic and immunologic responses in primates experimentally infected with *Onchocerca volvulus*. *Exp Parasitol* 1995; **80**:454-462.
46. Mehta K, **Chandrashekar, R**: Tranglutaminase activity in the microfilarial sheath. *Parasitology Today* 1995; **11**:254.
47. **Chandrashekar R**, Van Swinderen B, Taylor H, Weil J. Effect of ivermectin prophylaxis on antibody responses to *Onchocerca volvulus* recombinant antigens in experimentally infected chimpanzees. *Int J Parasitol* 1995; **25**:983.
48. Ramzy R, Helmy H, **Chandrashekar R**, Gad A, Faris R, Weil GJ. Evaluation of a recombinant antigen-based antibody assay for lymphatic filariasis in Egypt. *Ann Trop Med Hyg* 1995; **89**:443-446.
49. Singh RN, **Chandrashekar R**, Mehta K. Purification and partial characterization of transglutaminase from a dog filarial parasite, *Dirofilaria immitis*. *Int J Biochem Cell Biol* 1995; **27**:1285.
50. **Chandrashekar R**, Curtis KC, Li BW, Weil G. Molecular characterization of a *Brugia malayi* intermediate filament protein which is a major excretory-secretory product of adult worms. *Mol Biochem Parasitol 1995;* **73**:231.
51. Mehta K, **Chandrashekar R**, Rao UR. Transglutaminase-catalyzed incorporation of a host protein (p68) on the surface of *Brugia malayi* microfilariae. *Mol Biochem* Parasitol 1996; **76**:105.
52. Weil G, Ramzy RM, **Chandrashekar R**, Gad AM, Lowrie RC, Jr, Faris R. Parasite antigenemia without microfilaremia in bancroftian filariasis. *Am J Trop Med Hyg* 1996; **55**:333.
53. **Chandrashekar R**, Ogunrinade AF, Weil GJ. Use of recombinant *Onchocerca volvulus* antigens for diagnosis and surveillance. *Tropical Medicine and International Health* 1996; **1**:575.
54. **Chandrashekar R**, Curtis KC, Rawot BW, Kobayashi G, Weil GJ. Molecular cloning and characterization of a recombinant *Histoplasma capsulatum* antigen for diagnosis of human histoplasmosis. *J Clin Microbiol* 1997; **35**, 1071-1076.
55. Klimowski L, **Chandrashekar R**, Tripp CA. Cloning, expression and enzymatic activity of a thioredoxin peroxidase from *Dirofilaria immitis*. *Mol Biochem Parasitol* 1997; **90**:297.
56. **Chandrashekar, R**, Tsuji, T, Morales, T, Ozols, V, Mehta, K. An ERp60-like protein from the filarial parasite *Dirofilaria immitis* has both transglutaminase and protein disulfide isomerase activity. *Proc Natl Acad Sci USA* 1998; **95**:531.
57. Tsuji N, Morales T, Ozols V, Carmody A, **Chandrashekar R**. Molecular characterization of a calcium-binding protein from the filarial parasite *Dirofilaria immitis*. *Mol Biochem Parasitol* 1998; **97**:69.

58. **Chandrashekar R**, Kurtis, KC, Lu, W, Weil GJ. Molecular cloning of an enzymatically active thioredoxin peroxidase from *Onchocerca volvulus*. *Mol Biochem Parasitol* 1998; **93**:309.
59. Tsuji N, Morales T, Carmody A, Ozols V, **Chandrashekar R**. Identification of an asparagine amidohydrolase from the filarial parasite *Dirofilaria immitis*. *Int J Parasitol* 1999; **29**:1451.
60. Helmy H, Weil GJ, Faris R, Gad AM, **Chandrashekar R**, Ashour A, Ramzy RMR. Human antibody responses to *Wuchereria bancrofti* infective larvae. *Parasite Immunol* 2000; **22**:89.
61. **Chandrashekar R**, Tsuji N, Morales T, Carmody A, Ozols V, Welton J, Tang L. Cloning and characterization of a 1-Cys peroxiredoxin from the filarial parasite *Dirofilaria immitis*. *Parasitol Res* 2000; **86**:200.
62. Tsuji N, Morales T, Ozols V, Carmody A, **Chandrashekar R**. Cloning and preliminary Characterization of a novel cuticular antigens from the filarial parasite *Dirofilaria immitis*. *Parasitol Int* 2000; **49**:321.
63. Ogunrinade AF, Awolola SO, Rotimi O, **Chandrashekar R**. Longitudinal studies of skin microfilaria and antibody conversion rates in children living in an endemic focus of onchocerciasis in Nigeria. *J Trop pediatr* 2000; **46**:348.
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INVITED PUBLICATIONS

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Cloning and Characterization of Multigenes Encoding the Immunodominant 30-Kilodalton Major Outer Membrane Proteins of *Ehrlichia canis* and Application of the Recombinant Protein for Serodiagnosis

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A 30-kDa major outer membrane protein of *Ehrlichia canis*, the agent of canine ehrlichiosis, is the major antigen recognized by both naturally and experimentally infected dog sera. The protein cross-reacts with a serum against a recombinant 28-kDa protein (rP28), one of the outer membrane proteins of a gene (*omp-1*) family of *Ehrlichia chaffeensis*. Two DNA fragments of *E. canis* were amplified by PCR with two primer pairs based on the sequences of *E. chaffeensis omp-1* genes, cloned, and sequenced. Each fragment contained a partial 30-kDa protein gene of *E. canis*. Genomic Southern blot analysis with the partial gene probes revealed the presence of multiple copies of these genes in the *E. canis* genome. Three copies of the entire gene (*p30*, *p30-1*, and *p30a*) were cloned and sequenced from the *E. canis* genomic DNA. The open reading frames of the two copies (*p30* and *p30-1*) were tandemly arranged with an intergenic space. The three copies were similar but not identical and contained a semivariable region and three hypervariable regions in the protein molecules. The following genes homologous to three *E. canis* 30-kDa protein genes and the *E. chaffeensis omp-1* family were identified in the closely related rickettsiae: *wsp* from *Wolbachia* sp., *p44* from the agent of human granulocytic ehrlichiosis, *msp-2* and *msp-4* from *Anaplasma marginale*, and *map-1* from *Cowdria ruminantium*. Phylogenetic analysis among the three *E. canis* 30-kDa proteins and the major surface proteins of the rickettsiae revealed that these proteins are divided into four clusters and the two *E. canis* 30-kDa proteins are closely related but that the third 30-kDa protein is not. The *p30* gene was expressed as a fusion protein, and the antibody to the recombinant protein (rP30) was raised in a mouse. The antibody reacted with rP30 and a 30-kDa protein of purified *E. canis*. Twenty-nine indirect fluorescent antibody (IFA)-positive dog plasma specimens strongly recognized the rP30 of *E. canis*. To evaluate whether the rP30 is a suitable antigen for serodiagnosis of canine ehrlichiosis, the immunoreactions between rP30 and the whole purified *E. canis* antigen were compared in the dot immunoblot assay. Dot reactions of both antigens with IFA-positive dog plasma specimens were clearly distinguishable by the naked eye from those with IFA-negative plasma specimens. By densitometry with a total of 42 IFA-positive and -negative plasma specimens, both antigens produced results similar in sensitivity and specificity. These findings suggest that the rP30 antigen provides a simple, consistent, and rapid serodiagnosis for canine ehrlichiosis. Cloning of multigenes encoding the 30-kDa major outer membrane proteins of *E. canis* will greatly facilitate understanding pathogenesis and immunologic study of canine ehrlichosis and provide a useful tool for phylogenetic analysis.

Canine ehrlichiosis is caused by *Ehrlichia canis*, an obligatory intracellular bacterium. It was described originally in Algeria in 1935 (7), and it has now been reported throughout the world and at higher frequency in tropical and subtropical regions (13, 15, 32). Canine ehrlichiosis is characterized by fever, depression, anorexia, and weight loss in the acute phase, with laboratory findings of thrombocytopenia and hypergammaglobulinemia (3, 9). A subclinical phase follows the acute phase (5, 12, 28). In the chronic phase, in addition to the clinical signs and laboratory findings of the acute phase, hemorrhages, epistaxis, edema, and hypotensive shock may occur, which are often exacerbated by superinfection with other organisms (3, 9, 16).

Among several protein antigens of *E. canis*, the proteins in the 30-kDa range were shown to be dominant antigens and consistently recognized by sera from both experimentally and naturally infected dogs in Western blot analysis (14, 25, 26). The proteins of *E. canis* immunologically cross-react with *Ehrlichia chaffeensis* major antigens in the 30-kDa range (25). These *E. canis* and *E. chaffeensis* proteins were found to be major outer membrane proteins (OMPs) (22). Analysis of a 28-kDa major OMP (P28) gene of *E. chaffeensis*, one of the 30-kDa-range antigens, and its gene copies revealed that these proteins are encoded by a polymorphic multigene family (22). The rabbit serum against a recombinant *E. chaffeensis* P28 protein cross-reacted with the 30-kDa protein of *E. canis* (22).

Dot immunoblot assaying has been developed for serodiagnosis of several infectious agents (4, 10, 11, 30). The advantages of the assay are that an expensive instrument is not required and the interpretation of the results is easy, since positive and negative reactions can be distinguished by the naked eye. However, to be used as the antigen, purification of the organism from infected cells is essential, since *E. canis* is an obligate intracellular bacterium. Purification of *E. canis* is time-consuming and expensive, and serial passages of *E. canis*

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in the cell culture may produce batch-to-batch variations. Although, no genes of *E. canis* other than the 16S rRNA gene have thus far been identified, preparation of a recombinant major antigen is expected to greatly improve the serodiagnosis of *E. canis* infection.

In this study, three genes encoding the 30-kDa OMPs from the *E. canis* genome were identified. All were found to be homologous and phylogenetically characterized. A recombinant protein of *E. canis* which was expressed as a fusion protein was found to be highly antigenic. The dot immunoblot assay was developed with the recombinant *E. canis* protein.

MATERIALS AND METHODS

Organisms and purification. *E. canis* Oklahoma and *E. chaffeensis* Arkansas were cultivated in the DH82 dog macrophage cell line and purified by Percoll density gradient centrifugation (22) or Sephacryl S-1000 column chromatography (26).

PCR, cloning, and expression. The sequences of two forward primers, FECH1 and FECH2, were 5′-CGGGATCCGAATTCGG(A/T/G/C)AT(A/T/C)AA(T/C)GG(A/T/G/C)AA(T/C)TT(T/C)TA-3′ and 5′-CGGGATCCGAATTCTA(T/C)AT(A/T)AG(T/C)GG(A/T/G/C)AA(A/G)TA(T/C)ATG-3′, corresponding to amino acid positions 6 to 12 and positions 12 to 18, respectively, of the mature 28-kDa protein (P28) of *E. chaffeensis* (22). These primers have a 14-bp sequence (underlined) at the 5′ end to create an *Eco*RI site and a *Bam*HI site for insertion into an expression vector. The sequence of a reverse primer, REC1, was 5′-ACCTAACTTTCCTTGGTAAG-3′, complementary to the DNA sequence corresponding to amino acid positions 185 to 191 of the mature P28 of *E. chaffeensis* (22).

Genomic DNA of *E. canis* was isolated from Percoll gradient-purified organisms as described elsewhere (22). PCR amplification was performed by using a Perkin-Elmer Cetus DNA Thermal Cycler (model 480). The 0.6-kb products were amplified with both primer pairs, FECH1-REC1 and FECH2-REC1, and were cloned in the pCRII vector of a TA cloning kit (Invitrogen Co., San Diego, Calif.). The clones obtained by FECH1-REC1 and FECH2-REC1 were designated pCRII*p30* and pCRII*p30a*, respectively. Both strands of the insert DNA were sequenced by a dideoxy termination method with an Applied Biosystems 373 DNA sequencer.

For expression, the 0.6-kb fragment was excised from the clone pCRII*p30* by *Eco*RI digestion, ligated into *Eco*RI site of a pET29a expression vector, and amplified in *Escherichia coli* BL21(DE3)pLys (Novagen, Inc., Madison, Wis.). The clone (designated pET29*p30*) produced a fusion protein with 35-amino-acid and 21-amino-acid sequences carried from the vector at the N and C termini, respectively.

For purification of a recombinant P30 fusion protein (rP30), the cultivated clone was harvested at 4 h after induction with β-D-thiogalactopyranoside. The recombinant protein in the clone pET29*p30* was enriched in the pellet by three cycles of centrifugation of the lysate after disruption of the transformant by freezing-thawing and sonication. The final pellet was used as a partially purified rP30 antigen. Affinity-purified rP30 protein was obtained by chromatography with His-Bind Resin (Novagen, Inc.). Briefly, after preparation of the partially purified rP30 antigen, the insoluble protein was extracted with binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), including 6 M urea. After being applied to a Ni^{+}-conjugated column, the recombinant protein was eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 6 M urea. The refolding of the purified protein was achieved by sequential dialysis in 20 mM Tris-HCl (pH 7.9) containing 4 and 2 M urea and finally in 20 mM Tris-HCl buffer only and stored at −80°C until use.

Southern blot analysis. Genomic DNA extracted from the Percoll-purified *E. canis* (200 ng each) was digested with restriction enzymes, electrophoresed, and transferred to a Hybond-N^{+} nylon membrane (Amersham, Arlington Heights, Ill.) by a standard method (27). The 0.6-kb DNA inserts containing partial *p30* and *p30a* genes, cloned in pCRII*p30* and pCRII*p30a*, respectively, were separately labeled with [α-^{32}P]dATP by the random primer method with a kit (Amersham), and each labeled fragment was used for Southern blot analysis as a DNA probe. Hybridization was performed at 60°C in Rapid Hybridization buffer (Amersham) for 20 h. The nylon sheet was washed in 0.1× SSC (1× SSC containing 0.15 M sodium chloride and 0.015 M sodium citrate) with 1% sodium dodecyl sulfate (SDS) at 55°C, and the hybridized probes were exposed to Hyperfilm (Amersham) at −80°C.

Cloning and sequencing of 30-kDa protein gene copies from the *E. canis* genomic DNA. The *Hin*dIII DNA fragment, which was detected by genomic Southern blot analysis as described above, was inserted into pBluescript II KS(+) vectors, and the recombinant plasmids were introduced into *E. coli* DH5α. By using the colony hybridization method (27), two positive clones which contained ehrlichial DNA fragments of 3.6 and 7.3 kb were isolated with the ^{32}P-labeled inserts of pCRII*p30* and pCRII*p30a* as probes, respectively. DNA sequencing was performed with suitable synthetic primers by the dideoxy termination method described above.

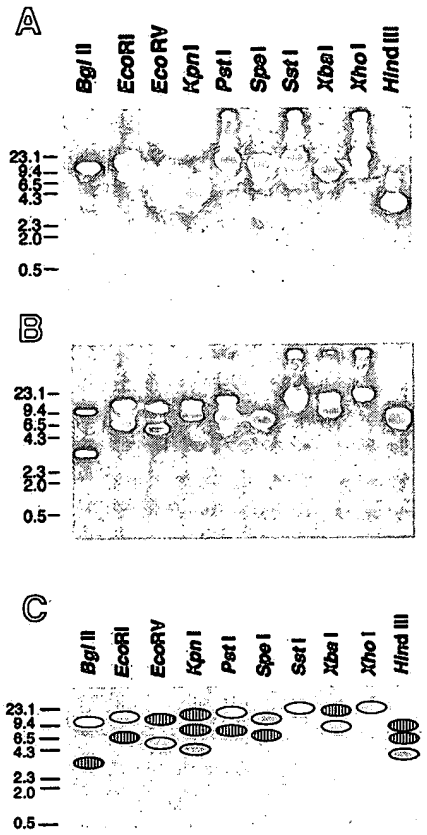

FIG. 1. Genomic Southern blot analysis of *E. canis* DNA with the partial *p30* gene probe (A) and with the partial *p30a* gene probe (B) and schematic representation of the blotting patterns (C). Numbers indicate molecular sizes in kilobases. Filled dots, bands hybridized with both p30 and p30a probes; striped dots, bands hybridized with p30a probe alone; lightly shaded dots, bands hybridized with p30 probe alone.

Sequence analysis. DNA and amino acid sequences were analyzed with the programs DNASIS (Hitachi Software Engineering America, Ltd., San Bruno, Calif.) and DNASTAR (DNASTAR Inc., Madison, Wis.). The amino acid sequences were aligned by using the CLUSTAL method in the DNASTAR program. Phylogenetic analysis was performed by using the PHYLIP software package (version 3.5) (8). An evolutionary distance matrix, generated by using the Kimura formula in the program PROTDIST in the package, was used for construction of a phylogenetic tree by using the unweighted pair-group method of analysis (8). The data were examined by using parsimony analysis (PROTPARS in the PHYLIP). A bootstrap analysis was carried out to investigate the stability of randomly generated trees by using SEQBOOT and CONSENSE in the same package.

Dog plasma and mouse serum. Totals of 34 and 8 dog blood samples with heparin or EDTA were obtained from the Southwest Veterinary Diagnostic Center (Phoenix, Ariz.) and at the Ohio State University Veterinary Teaching Hospital, respectively. All blood specimens collected were centrifuged at 250 × *g* for 5 min, and the plasma samples were used for this study. For Western blot analysis, these plasma samples were preabsorbed three times with pET29a-transformed *E. coli* at 4°C overnight prior to use. For preparation of the mouse anti-rP30 serum, a male mouse (BALB/c) was intraperitoneally immunized a total of four times at 10-day intervals, once with an equal mixture of the affinity-purified rP30 (30 μg of protein) and Freund's complete adjuvant (Sigma) and three times with an equal mixture of the protein (30 μg) and Freund's incomplete adjuvant. The mouse was sacrificed 7 days after final immunization, and the serum was prepared from blood collected from the heart.

IFA and Western blot analysis. Indirect fluorescent antibody assays, (IFA) and Western blot analysis were performed by a procedure described elsewhere (25). Fluorescein isothiocyanate-conjugated goat anti-dog immunoglobulin G (IgG; Organon Teknika Co., Durham, N.C.) and peroxidase-conjugated affinity-purified anti-dog IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) were used at dilutions of 1:200 for IFA and 1:2,000 for Western blot analysis, respectively, as secondary antibodies.

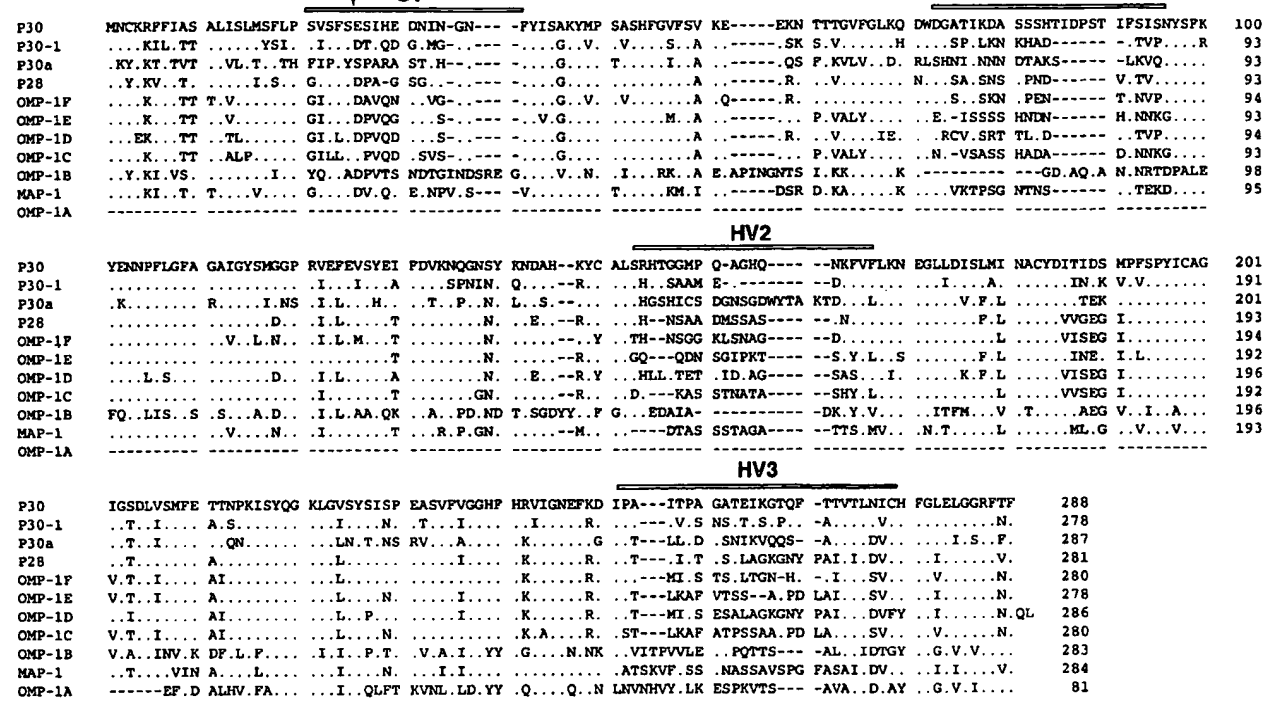

FIG. 2. Amino acid sequence alignment of P30, P30-1, and P30a of *E. canis*, seven members of *E. chaffeensis omp-1* multigene family (P28 and OMP-1A to OMP-1F), and MAP-1 of *C. ruminantium* (Senegal strain). The sequences of the *E. chaffeensis omp-1* gene family and MAP-1 are from the reports of Ohashi et al. (22) and Van Vliet et al. (31), respectively. Aligned positions of identical amino acids with P30 of *E. canis* are indicated by dots. Gaps (indicated by dashes) were introduced for optimal alignment of all proteins. Bars indicate an SV and three HVs (HV1, -2, and -3). The arrowhead indicate the putative cleavage site of the signal peptide.

Dot immunoblot assay. Protein concentrations of purified *E. canis* and recombinant rP30 antigens were determined by a bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as a standard. These antigens in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.4], 150 mM NaCl) were adsorbed onto a nitrocellulose membrane by using a dot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.), blocked for 30 min with TBS containing 2% milk, air dried, and stored at −20°C until use. For immunoassays, the antigen bound to a nitrocellulose strip was incubated with the plasma samples, which were diluted 1:1,000 in TBS containing 2% milk for 1 h at room temperature. After being washed three times with TBS containing 0.05% Tween 20 (T-TBS), the strip was incubated with peroxidase-conjugated affinity-purified anti-dog IgG (Kirkegaard) at a dilution of 1:2,000 in TBS containing 2% milk. After being washed with T-TBS, the antibody-bound dot was detected by immersing the strip in a developing solution (0.3% 3,3'-diaminobenzidine tetrahydrochrolide [Nacalai Tesque, Inc., Kyoto, Japan] and 0.05% hydrogen peroxide in 70 mM sodium acetate [pH 6.2]). The color intensity was analyzed by using background correction in image analysis software (ImageQuaNT program; Molecular Dynamics, Sunnyvale, Calif.).

GenBank accession number. The DNA sequences of the *p30*, *p30a*, and *p30-1* genes of *E. canis* have been assigned GenBank accession numbers AF078553, AF078555, and AF078554, respectively.

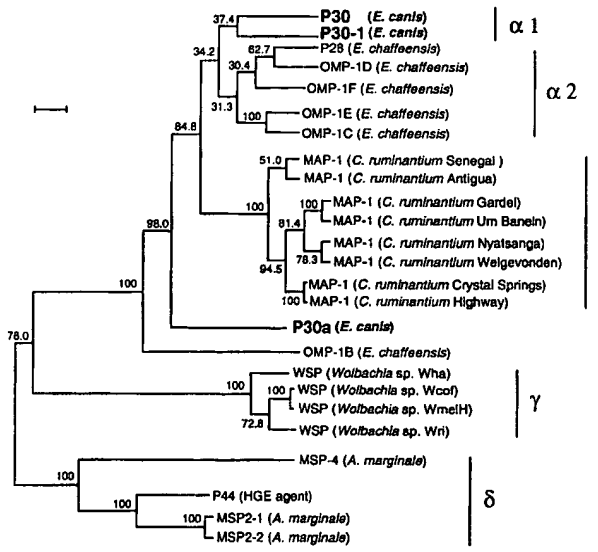

FIG. 3. Phylogenetic classification among P30, P30-1, and P30a of *E. canis* and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Evolutionary distance values were determined by the method described by Kimura, and the tree was constructed by the unweighted pair-group method of analysis. Scale bar indicates 10% divergence in amino acid sequences. Bootstrap values from 100 analyses are shown at the branch points of the tree. Bars with symbols indicate representative clusters. The GenBank accession numbers of the major OMP gene sequences of the organisms used in the analysis are as follows: P28 (*E. chaffeensis*), U72291; OMP-1B to OMP-1F (*E. chaffeensis*), AF021338; MAP-1 (*C. ruminantium* Senegal strain), I40882, MAP-1 (*C. ruminantium* Antigua strain), U50830; MAP-1 (*C. ruminantium* Gardel strain), U50832; MAP-1 (*C. ruminantium* Um Banein strain), U50835; MAP-1 (*C. ruminantium* Nyatsanga strain), U50834; MAP-1 (*C. ruminantium* Welgevonden strain), U49843; MAP-1 (*C. ruminantium* Crystal Springs strain), U50831; MAP-1 (*C. ruminantium* Highway strain), U50833; WSP (*Wolbachia* sp. Wha strain), AF020068; WSP (*Wolbachia* sp. Wcof strain), AF020067; WSP (*Wolbachia* sp. WmelH strain), AF020066; WSP (*Wolbachia* sp. Wri strain), AF020070; MSP-4 (*A. marginale*), Q07408; MSP2-1 (*A. marginale*), U07862; MSP2-2 (*A. marginale*), U36193; and P44 (HGE agent), AF059181.

RESULTS

Cloning and sequencing of three 30-kDa protein gene copies of *E. canis*. Two 0.6-kb DNA fragments containing partial *p30*

TABLE 1. Similarities among amino acid sequences of *E. canis* P30, P30-1, and P30a; *E. chaffeensis omp-1* family (OMP-1B to OMP-1F and P28); *C. ruminantium* MAP-1; *Wolbachia* spp. WSP; HGE agent P44; and *A. marginale* MSP-4, MSP2-1, and MSP2-2

Protein	% Amino acid sequence similarity and evolutionary distance for the following proteins[a]:										
	P30	P30-1	P30a	P28	OMP-1F	OMP-1E	OMP-1D	OMP-1C	OMP-1B	MAP-1 (Senegal)	MAP-1 (Antigua)
P30		80.2	70.8	80.6	80.5	78.6	77.8	77.5	63.2	75.4	76.2
P30-1	0.38628		71.6	79.8	81.7	78.7	78.3	77.3	63.2	74.7	75.6
P30a	0.60811	0.60559		73.9	72.1	73.3	71.2	72.1	58.8	67.2	67.8
P28	0.36288	0.40582	0.50899		85.7	82.3	86.3	81.1	63.6	76.4	77.5
OMP-1F	0.37862	0.36209	0.59907	0.27551		83.4	84.9	83.0	63.2	75.4	75.8
OMP-1E	0.41426	0.42866	0.52142	0.35465	0.32640		81.7	90.1	63.4	76.8	78.1
OMP-1D	0.45193	0.46724	0.61591	0.25793	0.28867	0.36288		81.5	63.2	73.5	74.5
OMP-1C	0.45426	0.48329	0.57469	0.39823	0.34577	0.18285	0.37688		62.4	76.0	77.5
OMP-1B	0.89214	0.87276	0.99793	0.81397	0.83501	0.82982	0.84498	0.89516		62.7	63.2
MAP-1 (Senegal)	0.50490	0.51605	0.76041	0.46987	0.50383	0.46987	0.57453	0.50564	0.92668		93.9
MAP-1 (Antigua)	0.47614	0.50899	0.74635	0.46755	0.52220	0.46096	0.57153	0.48952	0.88842	0.09122	
MAP-1 (Gardel)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.13499	0.11546
MAP-1 (Crystal Springs)	0.55702	0.53478	0.78883	0.52220	0.56563	0.49693	0.59089	0.53368	0.93601	0.13657	0.14142
MAP-1 (Highway)	0.52891	0.52047	0.76041	0.49443	0.54364	0.46987	0.57594	0.50564	0.93601	0.12383	0.12856
MAP-1 (Nyatsanga)	0.50593	0.49693	0.76544	0.49196	0.53368	0.46755	0.57296	0.48952	0.91855	0.13077	0.11963
MAP-1 (Um Banein)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.12658	0.11963
MAP-1 (Welgevonden)	0.52629	0.50383	0.74708	0.49877	0.53368	0.47419	0.60290	0.48952	0.92979	0.16080	0.14519
WSP (Wha)	1.57097	1.66864	1.78274	1.59949	1.50435	1.38174	1.61950	1.45510	1.41776	1.58338	1.48404
WSP (Wcof)	1.46262	1.62571	1.62571	1.55195	1.40877	1.29961	1.60271	1.41762	1.33110	1.55897	1.53089
WSP (WmelH)	1.48165	1.64952	1.64952	1.54244	1.39991	1.31514	1.59304	1.43572	1.34750	1.54961	1.49206
WSP (Wri)	1.46435	1.66864	1.70518	1.55687	1.46526	1.27219	1.57654	1.39076	1.32111	1.53292	1.47465
P44	1.77884	1.84928	2.04164	1.56146	1.74020	1.64702	1.64376	1.64702	1.64566	1.57894	1.63909
MSP-4	1.37226	1.39399	1.62744	1.38660	1.45473	1.36494	1.45413	1.47002	1.34294	1.23482	1.31702
MSP2-1	1.50323	1.53992	1.90757	1.40230	1.59474	1.53455	1.40877	1.50435	1.52758	1.53992	1.54847
MSP2-2	1.52476	1.53992	1.87540	1.40230	1.57132	1.53455	1.40877	1.50435	1.55019	1.51796	1.52616

[a] Values in the upper right half are percent amino acid sequence similarities; those in the lower left half are evolutionary distances.

and *p30a* genes, amplified by PCR, were cloned and sequenced as described in Materials and Methods. The 0.6-kb DNA, cloned in pCRII*p30*, had an open reading frame (ORF) of 579 bp encoding a 193-amino-acid protein with a molecular mass of 21,175 Da. Another 0.6-kb fragment, cloned in pCRII*p30a*, had an ORF of 564 bp encoding a 188-amino-acid protein with a molecular mass of 21,042 Da. The DNA and predicted amino acid sequences of the partial *p30a* gene were similar but not identical to those of the partial *p30* gene. Genomic Southern blot analysis of *E. canis* digested with several restriction enzymes revealed one and two DNA fragments which could strongly hybridize to the partial *p30* and *p30a* gene probes, respectively (Fig. 1). These restriction enzymes used do not cut within the *p30* and *p30a* gene probes, and, therefore, the result with the *p30a* probe indicates that another gene homologous to the *p30a* is present in the *E. canis* genome. In *Bgl*II, *Eco*RI, and *Pst*I digestion, the *p30* probe hybridized with the upper band of the two *p30a*-hybridized bands. In *Eco*RV and *Xba*I digestion, the *p30* probe hybridized with the lower band of the two *p30a*-hybridized bands. In *Kpn*I, *Spe*I, and *Hin*dIII digestion, the *p30* probe hybridized with one or two bands that were different from the *p30a*-hybridized bands.

Two DNA fragments of 3.6 and 7.3 kb were cloned by colony hybridization with the probes described above from the *Hin*dIII-digested genomic DNA of *E. canis*. Sequencing revealed a complete ORF of 864 bp for the *p30* gene in the 3.6-kb fragment and a complete ORF of 861 bp for *p30a* gene in the 7.3-kb DNA fragment. An additional ORF of 921 bp was found in the 3.6-kb DNA. The DNA sequence of the ORF (designated *p30-1*) was also similar but not identical to those of the *p30* and *p30a* genes. There are two potential start codons in the *p30-1* gene sequence. By comparison with the N-terminal amino acid sequences of *p30* and *p30a* genes, we chose a second ATG as a start codon for phylogenetic analysis. The coding region is 834 bp. The *p30-1* and *p30* genes were tandemly arranged with an intergenic space of 355 bp in the 3.6-kb fragment like the *E. chaffeensis omp-1* family (22). In addition to the result of the genomic Southern blot analysis, this finding showed that at least four homologous genes (*p30*, *p30-1*, *p30a*, and a gene homologous to *p30a*) exist in the *E. canis* genome, suggesting that these genes of *E. canis* are also encoded by a polymorphic multigene family as is the case with *E. chaffeensis* (22).

Structure of proteins encoded by *E. canis* multigenes. Three complete gene copies (*p30*, *p30-1*, and *p30a*) encode 278- to 288-amino-acid proteins with molecular masses of 30,485 to 31,529 Da. The 25-amino-acid sequence at the N termini of P30, P30-1, and P30a (encoded by *p30*, *p30-1*, and *p30a*, respectively) is predicted to be a signal peptide, as described previously (22). The molecular masses of the mature proteins calculated based on the predicted amino acid sequences are 28,750 Da for *p30*, 27,727 Da for *p30-1*, and 29,132 Da for *p30a*.

The predicted amino acid sequences of *E. canis* P30, P30-1, and P30a showed high similarity with those of members in the *E. chaffeensis omp-1* gene family (22) and that of major antigen protein 1 (MAP-1) of *Cowdria ruminantium* (31). These organisms are also serologically cross-reactive (6, 17, 18, 19, 20). The alignment of amino acid sequences of these proteins revealed substitutions or deletions of one or several contiguous amino acid residues throughout the molecules (Fig. 2). The significant differences in sequences among the proteins are observed in the regions designated SV (semivariable region) and HV (hypervariable region). Computer analysis for hydropathy revealed that protein molecules predicted for three *E. canis* gene copies contain alternative hydrophilic and hydrophobic motifs which are characteristic of typical transmembrane proteins. HV1 and HV2 were located in the hydrophilic regions (data not shown).

TABLE 1—*Continued*

% Amino acid sequence similarity and evolutionary distance for the following proteins:													
MAP-1 (Gardel)	MAP-1 (Crystal Springs)	MAP-1 (Highway)	MAP-1 (Nyatsanga)	MAP-1 (Um Banein)	MAP-1 (Welgevonden)	WSP (Wha)	WSP (Wcof)	WSP (WmelH)	WSP (Wri)	P44	MSP-4	MSP2-1	MSP2-2
76.4	74.5	75.4	75.8	76.4	75.2	44.4	44.6	44.4	44.4	19.5	45.6	27.8	27.4
74.7	73.9	74.3	74.7	74.7	74.5	44.0	45.1	44.8	44.6	20.5	47.6	29.3	29.1
67.6	65.9	66.5	66.7	67.6	67.2	41.5	43.2	42.9	42.5	19.5	43.1	24.2	24.2
75.8	74.5	75.4	75.2	75.8	74.9	44.0	44.8	44.8	44.6	22.5	46.9	29.7	29.5
74.5	73.3	73.9	73.9	74.5	73.9	44.6	45.9	45.9	45.3	21.1	46.2	27.8	27.8
76.2	75.4	76.2	76.0	76.2	75.8	45.7	46.9	46.7	46.9	22.0	47.5	28.2	28.0
74.1	73.1	73.5	73.3	74.1	72.4	43.6	44.2	44.2	44.2	22.0	46.0	29.9	29.7
75.8	74.5	75.4	75.6	75.8	75.6	45.3	46.1	45.9	46.1	22.0	46.6	28.6	28.4
63.6	63.2	63.2	63.2	63.6	62.9	45.5	45.1	44.8	45.5	19.1	45.8	26.9	26.5
91.4	90.7	91.4	91.6	91.8	90.1	44.6	45.1	45.1	45.1	21.8	48.8	28.0	28.0
91.8	90.7	91.4	91.6	91.6	90.3	44.8	45.1	45.3	45.3	21.8	48.0	28.0	28.0
	92.2	92.8	94.9	99.6	93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.12928		98.9	93.1	92.4	93.1	43.4	43.4	43.4	43.2	20.0	46.1	26.7	26.7
0.11692	0.01764		93.7	93.1	93.7	43.8	43.8	43.8	43.6	20.2	46.5	27.2	27.2
0.08788	0.11285	0.10076		94.5	95.4	43.8	43.8	43.8	43.8	20.5	46.7	28.0	27.8
0.00693	0.12514	0.11285	0.09570		93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.11966	0.11285	0.10076	0.08014	0.11966		44.2	44.0	44.0	44.0	20.2	46.5	27.8	27.6
1.51972	1.73099	1.65953	1.64538	1.51972	1.58048		86.1	86.1	90.3	12.5	42.5	22.9	22.7
1.47157	1.59304	1.53089	1.55897	1.47157	1.52893	0.27243		98.3	90.9	13.6	42.1	24.0	24.0
1.46262	1.58338	1.52153	1.54961	1.46262	1.51972	0.26757	0.03029		90.7	13.6	42.3	23.8	23.8
1.44526	1.64362	1.57654	1.53292	1.44526	1.50279	0.18429	0.17605	0.17691		13.6	43.2	24.0	23.8
1.62813	1.74020	1.71093	1.68253	1.62813	1.71093	2.06354	2.15803	2.14440	2.09032		25.7	45.5	45.2
1.33120	1.35101	1.30992	1.31112	1.33120	1.33120	1.72157	1.96007	1.90199	1.72157	1.20170		35.6	34.9
1.50996	1.57836	1.53304	1.46817	1.50996	1.48884	1.70865	1.79325	1.81891	1.72741	0.83164	1.20880		95.6
1.50996	1.55543	1.51116	1.46817	1.50996	1.48884	1.70865	1.75923	1.78382	1.72741	0.84284	1.23930	0.05064	

Phylogenetic relationship among the three *E. canis* 30-kDa proteins and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Recently, several major OMP genes which are closely related to the *E. canis* 30-kA protein have been cloned from rickettsiae (2, 21–24, 31, 34). The phylogenetic tree consisting of 25 major OMPs of the organisms including P30, P30-1, and P30a of *E. canis* was constructed from the estimated evolutionary distances (Fig. 3). The overall pattern of the tree reflects the result based on 16S rRNA gene sequence analysis of the rickettsiae. The 23 representatives, except for *E. canis* P30a and *E. chaffeensis* OMP-1B, are divided into four groups as follows: *E. canis* and *E. chaffeensis*, group α; *C. ruminantium*, group β; *Wolbachia* sp., group γ; and the agent of human granulocytic ehrlichiosis (HGE) and *Anaplasma marginale*, group δ. Group α formed a subcluster of *E. canis* P30 and P30-1 (group α1), which was separated from another subcluster composed of five *E. chaffeensis* OMPs (group α2). The similarities between P30 and P30-1 of *E. canis* in group α1, between groups α1 and α2, between groups α1 and β, between groups α1 and γ, and between groups α1 and δ were 80.2%, 77.3 to 80.6%, 73.9 to 76.4%, 44.0 to 45.1%, and 19.5 to 47.6%, respectively (Table 1). On the other hand, *E. canis* P30a and *E. chaffeensis* OMP-1B were far from group α and were located between groups β and γ. The similarities between *E. canis* P30a and group α1, between P30a and group α2, between P30a and group β, between P30a and group γ, and between P30a and group δ were 70.8 to 71.6%, 71.2 to 73.9%, 65.9 to 67.8%, 41.5 to 43.2%, and 19.5 to 43.1%, respectively.

Expression of the *E. canis p30* gene. The clone pET29*p30* produced a 249-amino-acid fusion protein with a molecular mass of 27,316 Da (Fig. 4A). The recombinant protein (rP30) with minimum *E. coli* contamination detectable was obtained in the pellet by centrifugation of the lysate of the transformant (Fig. 4B [partially purified antigen]). The rP30 protein further purified by affinity chromatography from this preparation had a single band on SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 4B [affinity-purified antigen]). The immunoreactions of *E. canis* rP30 with a total of 42 clinical dog plasma specimens were examined. The IgG-IFA titers of 29 plasma samples were 1:20 to 1:10,480. The remaining plasma samples were IFA negative (<1:20). Western blot analysis revealed that all IFA-positive plasma samples recognized the partially purified rP30 fusion protein (27 kDa) and a 30-kDa protein of

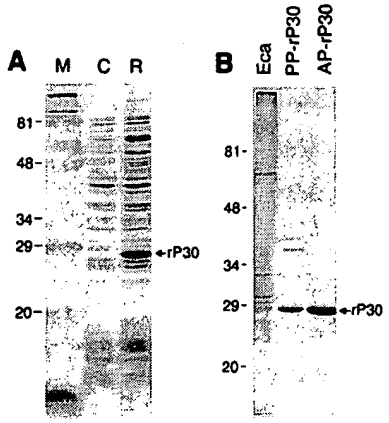

FIG. 4. SDS-PAGE profiles of a recombinant clone expressing P30 of *E. canis* (A) and the purified recombinant protein (B). Gels were stained with Coomassie blue. Lanes: M, molecular size markers; C, pET29-transformed *E. coli* (negative control); R, pET29*p30*-transformed *E. coli* (recombinant); Eca, purified *E. canis*; PP-rP30, partially purified rP30 fusion protein of *E. canis*; and AP-rP30, affinity-purified rP30 fusion protein. The recombinant rP30 protein is indicated by the arrow. The numbers on the left of each panel indicate molecular masses in kilodaltons.

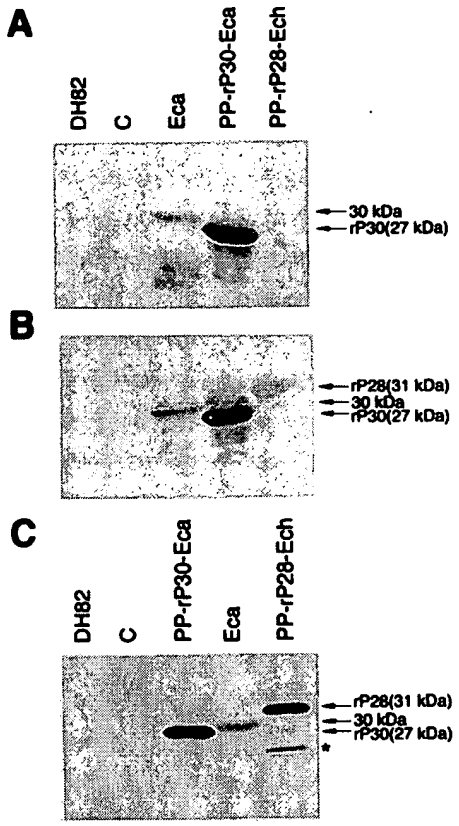

FIG. 5. Western blot analysis with clinical dog plasma with canine ehrlichiosis (A and B) and mouse anti-rP30 serum (C). (A) Dog plasma with a 1:40 IFA titer against *E. canis*; (B) dog plasma with a 1:1,280 IFA titer. Lanes: DH, DH82 dog macrophage cell (negative control); C, a pET29-transformed *E. coli* (negative control); Eca, purified *E. canis* (reactive 30-kDa protein is indicated by arrows in each panel); PP-rP30-Eca, a partially purified rP30 fusion protein (27 kDa) of *E. canis*; and PP-rP28-Ech, a partially purified rP28 fusion protein (31 kDa) of *E. chaffeensis* (22). Another smaller reactive band which may be a degradation product of rP28 of *E. chaffeensis* is indicated by an asterisk.

purified *E. canis* (one of the blots is shown in Fig. 5A), but none of 13 negative plasma samples reacted with any proteins of partially purified rP30 and purified *E. canis* (data not shown). Eight of the 29 positive plasma samples reacted weakly with recombinant P28 fusion protein (rP28 [31 kDa]) of *E. chaffeensis* (22) (one of the blots is shown in Fig. 5B), but the remaining plasma samples did not. A mouse anti-rP30 serum which was prepared by immunization with the affinity-purified antigen reacted with the rP30 antigen, a 30-kDa protein of purified *E. canis*, and an rP28 of *E. chaffeensis* (Fig. 5C). Another smaller band which was observed with *E. chaffeensis* rP28 may be a degradation product of rP28 (asterisk in Fig. 5C), since the plasma sample did not react with *E. coli* proteins. These results showed that rP30 of *E. canis* is highly antigenic and that the antigenic epitope is expressed.

Dot immunoblot assay with the purified whole organism antigen and the recombinant antigen. (i) Optimum amount of antigen per dot. Western blot analysis and dot immunoblot assaying in the preliminary experiments supported the interpretation that there are no significant differences between affinity-purified and the partially purified rP30 in specificity and sensitivity (data not shown). If partially purified recombinant protein is suitable for serodiagnosis, it will be more cost-effective. By dot immunoblot assaying we examined in detail whether partially purified rP30 is suitable as an antigen for serodiagnosis.

Nitrocellulose strips having serially diluted purified *E. canis* or partially purified rP30 antigen of *E. canis* were reacted at a 1:1,000 dilution with dog plasma samples with different IFA titers against *E. canis*, and the color intensities of the reaction of each dot were compared (Fig. 6). Dots of 0.01 to 1 μg of the purified organisms (Fig. 6A) or dots of 0.025 to 1 μg of rP30 (Fig. 6B) that reacted with positive plasma samples (>1:20 in IFA titer) were clearly distinguishable from those that reacted with negative plasma samples (<1:20) by the naked eye. There was no nonspecific reaction with the negative plasma samples when purified *E. canis* was used as an antigen; however, a weak nonspecific reaction with IFA-negative plasma was observed in dots of 0.25 to 1 μg of partially purified rP30 antigen. Based on these results, the optimum amounts of antigens per dot were determined to be 1 and 0.5 μg for antigen proteins of purified *E. canis* and partially purified rP30, respectively. These results show that the partially purified recombinant protein is apparently sufficient as an antigen for serodiagnosis.

(ii) Optimum dilution of antiserum. The immunoreactivities of plasma at dilutions of 1:300, 1:1,000, and 1:3,000 were examined with nitrocellulose strips of the purified *E. canis* an-

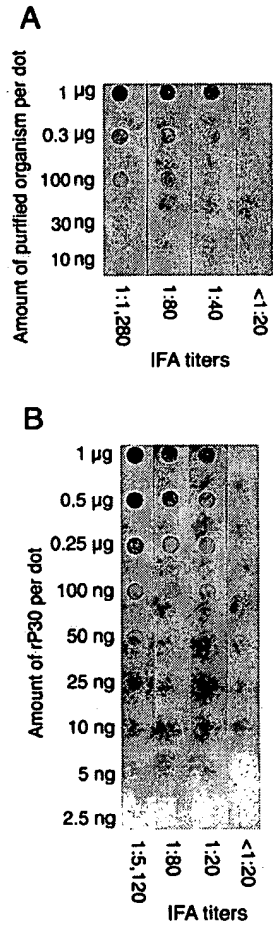

FIG. 6. Optimum amount of antigens for dot blot assaying with purified *E. canis* antigen (A) or partially purified rP30 antigen (B). Purified organism antigen (10 ng to 1 μg) or rP30 antigen (2.5 ng to 1 μg) was blotted onto the nitrocellulose sheet, reacted with each plasma at a 1:1,000 dilution as primary antibody, and reacted with secondary antibody (peroxidase-conjugated affinity-purified anti-dog IgG antibody) at a 1:2,000 dilution.

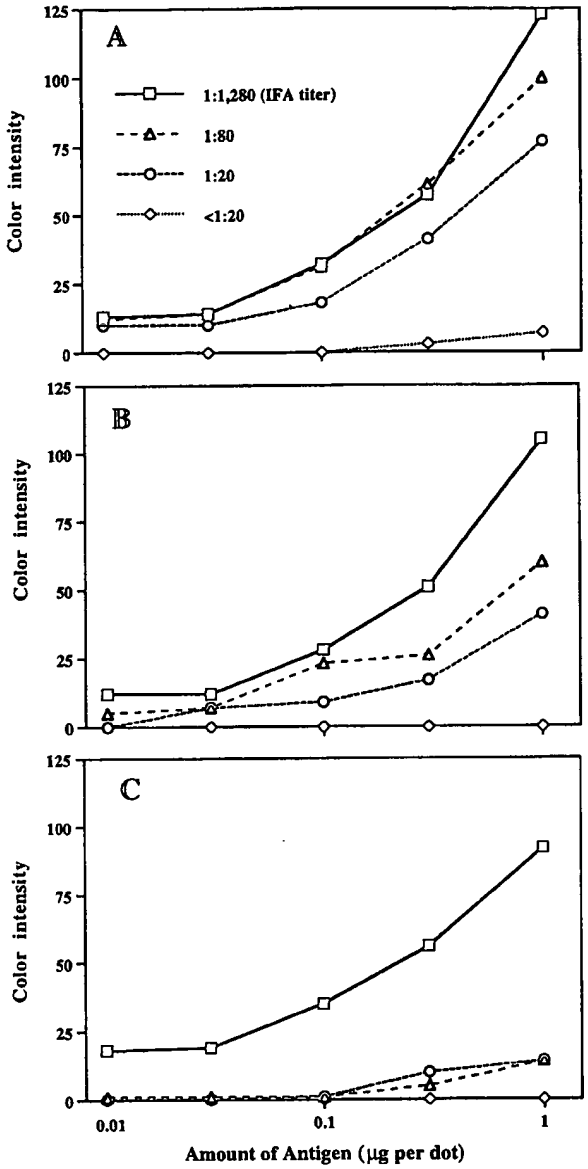

FIG. 7. Optimum plasma dilutions for dot blot assay. Purified *E. canis* antigen was blotted as described in the legend to Fig. 6. The antigens were incubated with plasma at dilutions of 1:300 (A), 1:1,000 (B), and 1:3,000 (C). The plasma samples used were the same as those used for Fig. 6A. The color intensity of each dot was determined by using the image software program (ImageQuaNT).

tigen as shown in Fig. 6A. The color intensity values were plotted in graphs (Fig. 7). At a 1:300 dilution (Fig. 7A), color development occurred in the dots having an antigen greater than 0.3 μg per dot with IFA-negative plasma. At a 1:3,000 dilution (Fig. 7C), color intensities of all plasma samples were low, especially in the case of positive plasma samples with low IFA titers (1:20 and 1:80). At a 1:1,000 dilution (Fig. 7B), positive plasma with even the lowest IFA titer (1:20) was distinguishable from IFA-negative plasma by the naked eye, especially with 1 μg of purified *E. canis* antigen per dot (Fig. 6A). The optimum dilution of plasma for testing was, therefore, 1:1,000.

(iii) Examination of clinical dog plasma with purified *E. canis* and partially purified rP30 antigens. A total of 42 clinical dog plasma samples were examined with 1 μg of purified *E. canis* antigen per dot and 0.5 μg of partially purified rP30 antigen per dot (Fig. 8). The plasma samples with higher IFA titers showed a darker reaction with both native and recombinant antigens. The color intensities between plasma with IFA titers of >1:20 and IFA-negative plasma were clearly distinguishable by the naked eye. The correlation between IFA titers and color intensity values by the dot immunoblot assay was examined (Fig. 9). The maximum color intensity values of 13 IFA-negative plasma samples (<1:20) were zero (background) in the purified *E. canis* antigen and 10 in the rP30 antigen. All 29 IFA-positive plasma samples (>1:20) showed color intensity values of greater than 19 in the purified *E. canis* and 18 in the rP30 antigen. The highest color intensity values were 105 in the purified organism and 114 in the rP30 antigen. In both native and recombinant antigens, color intensity values correlated with IFA titers. The correlation coefficients between IFA titers and color intensities of native and recombinant antigens were 0.71 ($P < 0.001$) and 0.68 ($P < 0.001$), respectively. Therefore, it may be possible to estimate an approximate titer of the test serum or plasma by comparing the color densities with those of serially diluted standard serum or plasma.

DISCUSSION

The availability of recombinant immunodominant major surface proteins of *E. canis* will greatly assist in diagnosis and in understanding of the pathogenesis of this intracellular bacterium, such as invasion of host cells, elicitation of the immune response, and mechanisms of the clinical disease. The 30-kDa protein of *E. canis* was shown to be the immunodominant major OMP, which can be recognized by naturally and experimentally infected dog sera (14, 25, 26). Therefore, the 30-kDa protein is the primary recombinant antigen candidate for use in the serodiagnosis of *E. canis* infection. The present study is the first report of molecular characterization of 30-kDa major OMPs of *E. canis*.

Polymorphic multigene families encoding the major OMPs have been identified in *E. chaffeensis*, the HGE agent, and *A. marginale*, which are closely related to *E. canis* based on 16S rRNA gene sequences. Six copies of the *E. chaffeensis p28* gene (*omp-1* gene family) are tandemly arranged with intergenic spaces (22), while copies of the HGE agent *p44* gene and the *A. marginale msp-2* and *msp-3* genes are distributed widely throughout the genomes (1, 23, 34). In this study, the 30-kDa proteins of *E. canis* were also shown to be encoded by a polymorphic multigene family. The two *E. canis* genes are tandemly arranged with an intergenic space as are members of the *E. chaffeensis omp-1* gene family. Although we demonstrated the presence of four gene copies of 30-kDa *E. canis* proteins in the genome, additional gene copies which are tandemly arranged may exist in three genomic *Hin*dIII DNA fragments which hybridized to *p30* and *p30a* probes. Sequence analysis revealed that the 30-kDa proteins (P30, P30-1, and P30a) of *E. canis* had characteristics of the *E. chaffeensis* OMP-1 family (22) and *C. ruminantium* MAP-1 (31). The *C. ruminantium* MAP-1 has been reported to be cross-reactive to a 27-kDa protein of *E. canis* (19), although it is unknown whether the 27-kDa protein is identical to P30, P30-1, or P30a of *E. canis* in this study. Phylogenetic analysis based on the homologs from the closely related rickettsiae revealed that P30 and P30-1 of *E. canis* are present in the same cluster but that P30a is far from the cluster, suggesting that the multigenes encoding the 30-kDa *E. canis* proteins are widely divergent. Interestingly, in the phylogenetic tree, the 30-kDa *E. canis* proteins, the *E. chaffeensis* OMP-1 family, the HGE agent P44, and *A. mar-*

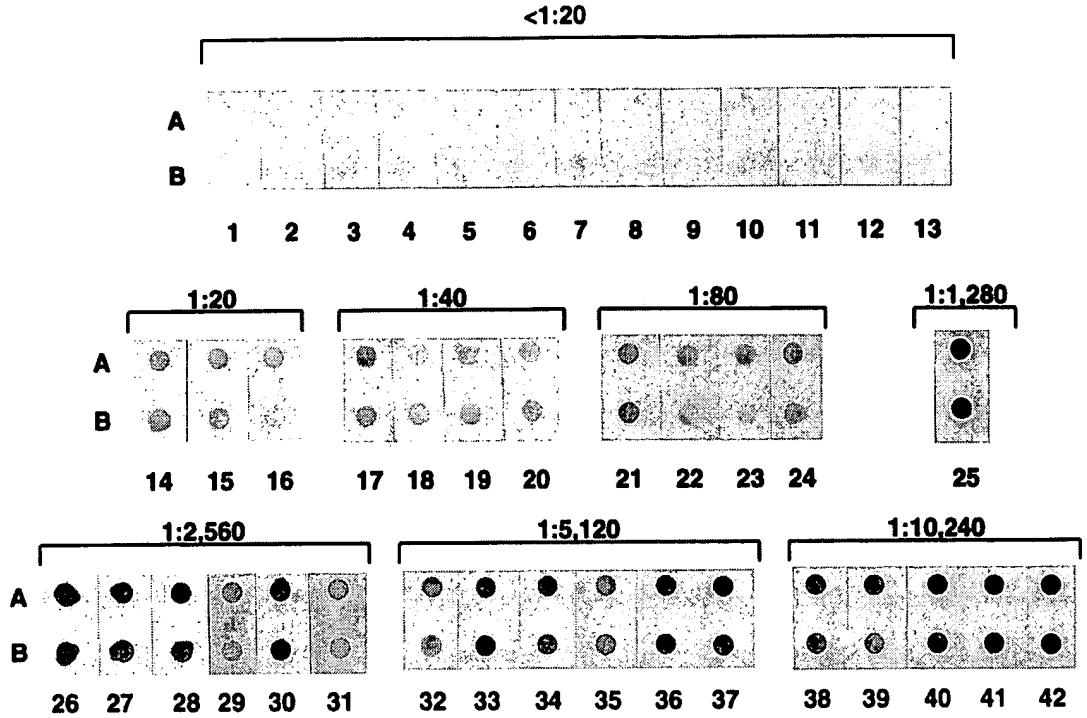

FIG. 8. Reaction profiles of purified *E. canis* antigen (1 μg) (A) and partially purified rP30 antigens (0.5 μg) (B) with 42 plasma samples. Plasma identifications are indicated below each dot. Numbers above brackets indicate the IFA titers of the plasma samples.

ginale MSP-2 are encoded by a polymorphic multigene family as described above. However, *C. ruminantium* MAP-1, *Wolbachia* sp. WSP, and *A. marginale* MSP-4 are encoded by a single gene (2, 21–24, 31). The diversities reported among the *C. ruminantium* MAP-1s and among the *Wolbachia* sp. WSPs are strain variation (2, 24, 31).

Molecular analysis of *E. canis* 30-kDa antigens such as ours is important in understanding the antibody responses of animals, because the antigenic diversity may influence the specificity and sensitivity of the serologic assay. Previously, we observed in the Western blot analysis that acute-phase serum (before 30 days postinoculation) from an *E. canis*-infected dog reacted strongly with a 30-kDa protein but weakly with a 31-kDa protein. However, the reactivity of the chronic-phase serum (after 60 days postinoculation) from the same dog was reversed (strong reaction with the 31-kDa protein and weak reaction with the 30-kDa protein) (14). This might be due to differential expression of the multigene encoding the 30-kDa protein of *E. canis* during infection. Although it is unknown whether the genes of P30, P30-1, and P30a were expressed by *E. canis* in tissue culture or in the infected dog, the recombinant P30 protein constructed in this study expressed the antigenic epitope which can react with all IFA-positive dog plasma samples used, suggesting that the antigenic epitope conserved among the 30-kDa protein gene family is expressed. This strongly supports the idea that rP30 is useful as an antigen for serodiagnosis of canine ehrlichiosis.

For serodiagnosis of canine ehrlichiosis, IFA is widely used. However, a fluorescence microscope and trained personnel are required for this test. Furthermore, cell culture of *E. canis* may produce batch-to-batch variation. A consistent and simple assay that can detect specific antibodies without expensive equipment would be an invaluable aid in serodiagnosis. In the dot immunoblot assay, antibody-positive serum can be distinguished from antibody-negative serum by the naked eye, and if proper color standards are provided, anyone can easily make the final evaluation. The greatest obstacle for the development of this assay is the production of diagnostic antigens sufficient in purity and amount. If recombinant antigens are available, the antigen preparation would be simpler, more consistent, and economical than purified organism antigen preparation. Previously, a dot blot enzyme-linked immunoassay for detecting antibodies to *E. canis* has been reported (4). However, the crude antigens, freed from host cells by freezing-thawing, were used in that study. Neither recombinant antigens nor the purified antigens (such as organisms purified by Sephacryl S-1000 column chromatography) were used. Additionally, that report contains only one page of description without any data. Therefore, we think our dot immunoblot assay using the recombinant 30-kDa antigen of *E. canis* would greatly enhance serodiagnosis of canine ehrlichiosis.

Recognition of the lowest positive IFA titer (1:20) plasma by a dot immunoblot assay with 1 μg or less of protein of the whole organism or the recombinant antigen per dot shows that this assay is as sensitive as IFA. Although the specificity of the test, except for cross-reactivity with *E. chaffeensis*, was not analyzed in this study, as with any other serologic test, dot immunoblot assaying probably cannot distinguish among antigenically cross-reactive members of the tribe *Ehrlichieae*. However, the use of recombinant *E. canis* antigen gave greater sensitivity than the use of recombinant *E. chaffeensis* antigen for serodiagnosis of canine ehrlichiosis. Western blot analysis revealed that 8 of 22 IFA-positive plasma samples slightly cross-reacted with recombinant 28-kDa protein of *E. chaffeensis*. This weak cross-reactivity is not a potential problem for clinics, since treatment is the same for all of the ehrlichial agents.

In dot immunoblot assays of 29 IFA-positive plasma sam-

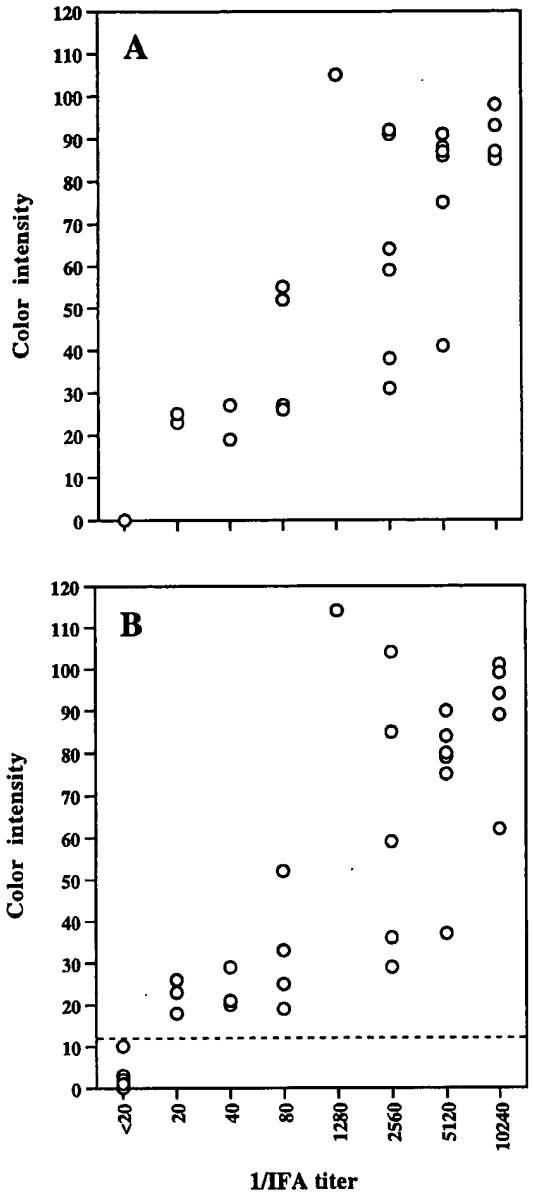

FIG. 9. Correlation between IFA titer (reciprocal dilutions) and color intensity of the dot immunoassay with purified *E. canis* antigen (A) and partially purified rP30 antigen (B). The color intensities of all dots in Fig. 8 were determined and plotted. Each circle represents one plasma specimen ($n = 42$). The correlation coefficients were 0.71 ($P < 0.001$) for graph A and 0.68 ($P < 0.001$) for graph B. The dashed line in graph B represents the cutoff value, which was determined from the highest color intensity in the immunoreaction with 13 negative plasma samples.

ples, 5 had color intensities of the purified organism antigen greater or lesser than those of the recombinant antigens. Additional major immunodominant proteins of *Ehrlichia* spp. are heat shock proteins (HSPs) (29, 33). Consequently, when anti-HSP antibody or antibody against protein antigen other than P30 is present in the plasma, whole organism antigens would give an immunoreaction stronger than that of the recombinant protein. On the contrary, when anti-P30 antibody is dominant in the plasma, the reaction with the recombinant protein would be stronger than that with the whole organism antigen. More importantly, the recombinant antigen-dot blot assay could clearly detect all of the 29 IFA-positive plasma samples. Furthermore, between native and recombinant antigens, no significant difference was observed in the correlation coefficient between IFA titers and the blot color intensity. Therefore, the rP30 antigen-immunodot blot assay offers advantages over the other serodiagnostic tests in general availability, ease of handling, and accuracy in the serodiagnosis of *E. canis* infection. Additionally, although it was not described in this paper, this *E. canis* recombinant antigen can be applied to enzyme-linked immunosorbent plate assays or other serodiagnostic assays as well.

ACKNOWLEDGMENT

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